Exposure of Corn Plants and Grass to RN1014

Frédéric D'Aoust, Luis Ortega, Olivier Landry and Glen Austin

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Objective: The objective of this study was to ascertain the effect of the genetically enhanced yeast strain RN1014 on growth of plants under laboratory conditions.

Executive Summary

The genetically enhanced *Saccharomyces cerevisiae* strain RN1014, with the ability to ferment xylose and hence applicability in the second generation fuel ethanol arena, was tested for its environmental effects. Grass and maize were grown in the lab and exposed to the RN1014 strain. The plants were chosen on the basis of ease of manipulation, material accessibility and most likely source of accidental exposure due to the close proximity to fuel ethanol plants in the industrial environment. Preliminary plant assays demonstrated no detectable impact of RN1014 application on plant growth of either lawn grass or maize plants

1.0 Background

Royal Nedalco has isolated one of the most promising pentose fermenting *S. cerevisiae* strains to date with the use of a combination of genetic and evolutionary engineering (Kuyper *et al.*, 2003). The heterologously constructed strain expresses a gene cloned from an obligate anaerobic fungus isolated from elephant dung. Under laboratory conditions this strain has demonstrated the ability to co-ferment glucose and xylose.

The pentose fermenting yeast strain, RN1014 used in this study was constructed and provided by Royal Nedalco (Bergen Op Zoom, NL). The *S. cerevisiae* strain CEN.PK102-3A (*MATa ura3-52 leu2-112*) was genetically modified to obtain a strain that has the ability to efficiently ferment xylose. The initial strain, designated RWB218, contained plasmid sequences and antibiotic resistance markers (Kuyper *et al.*, 2005). Chromosomal integration is considered a prerequisite to render industrial organisms genetically stable, which can not be obtained with multi-copy plasmid-carrying strains (Zhang *et al.*, 1996). To make this strain more suitable for industrial use, all unwanted sequences were removed. The plasmid borne genes were integrated into the chromosome and the antibiotic markers used during cloning were excised. The resulting marker free strain designated RN1001 was used for the isolation of the diploid variant RN1014.

2.0 Material and Methods

2.1 Planting of corn and grass seed

A total of three separate seed trays were prepared with seed starter mix (PlantSmart™; Wal-Mart Canada Inc. Mississauga, ON). The mix was placed to an appropriate depth (1.5 to 2 inches) in each tray as to fill the available volume. The trays were labelled as: (i) control, (ii) leaf treatment and (iii) root treatment. In one half of each tray grass seeds were planted by layering grass seeds over the surface of the plant mix and lightly covering the seeds with additional seed starter mix. In the second half of each tray maize seeds were planted 4 inches from each other in two rows at a depth of one half inch (Figure 1).

The seed trays were placed in a fume hood equipped with a F032/765/ECO OCTRAN® fluorescent lamp (Osram Sylvania Ltd., Mississauga, ON.) suspended above the trays along the central line of the three trays at a height of two inches above the seed tray (Figure 1). The height was increased after shoot emergence.

2.2 Growth and collection of RN1014

The yeast strain evaluated in this study was obtained from the Lallemand Yeast Culture Collection (LYCC). Yeast inoculums were prepared from overnight cultures grown in 500 mL YP+10%D complete medium (10 g/L yeast extract, 20 g/L peptone, 100 g/L glucose) from single pure colonies. The cultures

were incubated at 32°C with agitation (150 rpm) for 24 hours using an Innova-40 incubator shaker (New Brunswick Scientific Co. Inc., Edison, NJ).

Propagated cells were washed with 0.85% saline solution and subsequently resuspended in 250 mL 0.85% saline solution at a cell concentration of 1 x 10^7 cells/mL, Cell quantification was determined by microscopic observation using a hemocytometer.

2.3 Application of RN1014 to plant material

The seed and plant mix trays labelled (i) and (ii) were watered with sterile saline. Tray (iii) was watered with an equal volume of saline into which the organism RN1014 was added at a concentration of 1 x 10^7cells/mL. The trays were watered daily depending on need (soil was kept moist but not overly wet). Digital photographs were taken to record the plant growth.

After emergence of plants in tray (ii), a volume of 2 mL saline containing the organism RN1014 at a concentration of 1 x 10^7cells/mL was applied to the plant foliage with an atomizer. This was accomplished remotely from the other trays. This program was continued for seven days after emergence. Digital photographs were taken to record the plant growth.
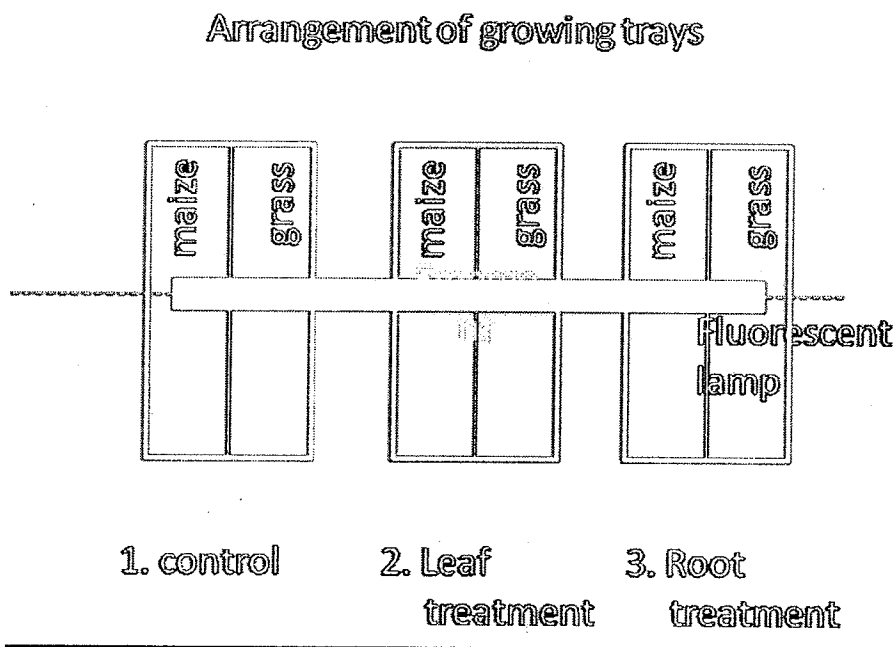
Arrangement of growing trays

1. control   2. Leaf treatment   3. Root treatment

Figure 1. Schematic representation of the arrangement of growing trays.

3.0 Results and Discussion

A series of plant assays were conducted under laboratory conditions to determine the environmental impact of the genetically enhanced RN1014 yeast strain. The plant assays were conducted using two grass species, lawn grass and maize. The plants were chosen on the basis of ease of manipulation, material accessibility and most likely source of accidental exposure due to the close proximity to fuel ethanol plants in the industrial environment. The influence on growth was established using three treatment conditions: leaf application, root application and a non application control.

3.1 Impact of RN1014 on the growth of lawn grass

The potential effect of exposure to the genetically enhanced yeast strain RN1014 was determined on the growth of lawn grass. The plant roots or leaves were treated with a saline solution containing the RN1014 yeast strain. The impact on growth was determined by visual inspection of the plant at regular time intervals. No observable differences could be detected between the treated grass and the inoculated control after 7 days (Figure 2) and 30 days (Figure 3).
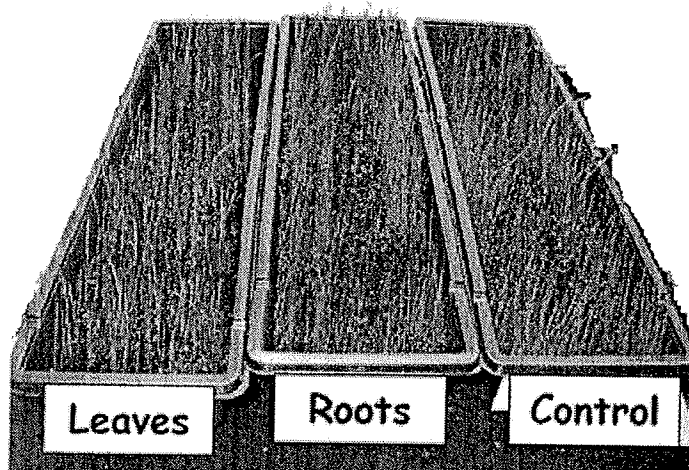
Figure 2. Photograph of lawn grass exposed to the genetically enhanced yeast RN1014. A saline solution containing RN1014 was used to inoculate the leaves or roots of grass plants. The control treatment consisted of uninoculated grass plants. Grass plants were grown for 7 days post-inoculation.
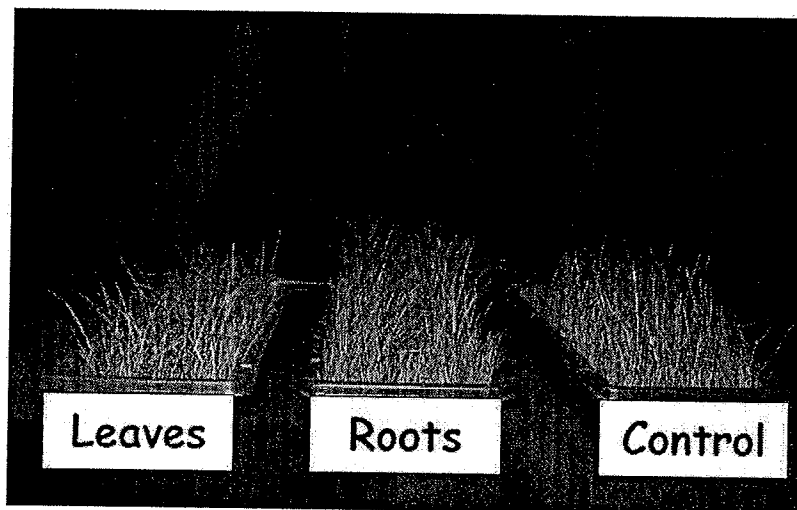
Figure 3. Photograph of lawn grass exposed to the genetically enhanced yeast RN1014. A saline solution containing RN1014 was used to inoculate the leaves or roots of grass plants. The control treatment consisted of uninoculated grass plants. Grass plants were grown for 30 days post-inoculation.

3.2 Impact of RN1014 on the growth of maize

The potential environmental impact of accidental exposure of maize root and leaves to the genetically enhanced yeast strain RN1014 was determined using plant assays. The plant roots and leaves were treated with a saline solution containing the RN1014 yeast strain. The impact on growth was determined by visual inspection of the plant at regular time intervals. No observable differences could be detected between the treated maize and the inoculated control after 11 days (Figure 4) and 21 days (Figure 5). Under all three conditions tested including the inoculated control, the maize plants started to show signs of nitrogen deficiencies after 21 days. This was not unexpected since the plants were only irrigated with saline without the addition of additional nitrogen.
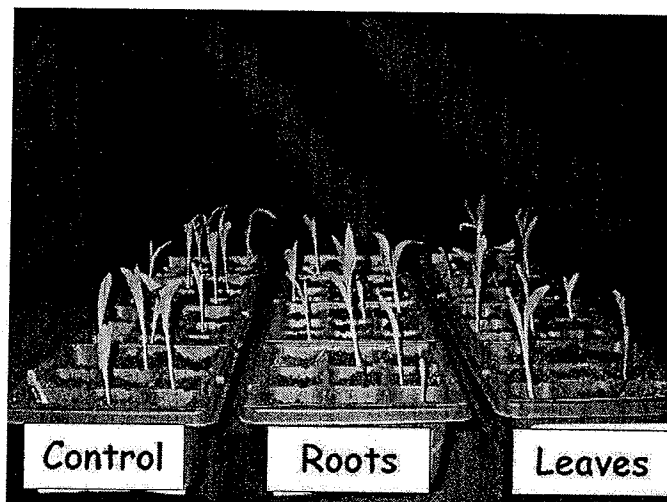

Figure 4. Photograph of maize exposed to the genetically enhanced yeast RN1014. A saline solution containing RN1014 was used to inoculate the leaves or roots of grass plants. The control treatment consisted of uninoculated grass plants. Grass plants were grown for 11 days post-inoculation.

Figure 5. *Photograph of maize exposed to the genetically enhanced yeast* RN1014. *A saline solution containing* RN1014 *was used to inoculate the leaves or roots of grass plants. The control treatment consisted of uninoculated grass plants. Grass plants were grown for 21 days post-inoculation.*
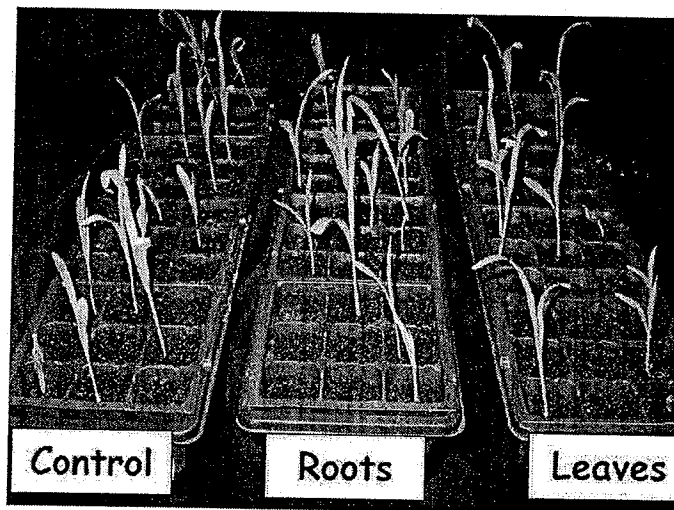
4.0 Conclusions

Preliminary plant assays demonstrated no detectable impact of RN1014 application on plant growth of either lawn grass or maize plants. Under all treatment conditions evaluated the lawn grass appeared normal and healthy. Similar results were obtained with maize although all plants started to show signs of nitrogen deficiencies towards the end of the experiment. The results represented in this report should help support the claim that the genetic enhanced yeast strain RN1014 poses no environmental danger and should be considered safe.

5.0 References

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Saccharomyces cerevisiae, a potential pathogen towards grapevine, *Vitis vinifera*

Sabine Gognies [b], Abdel Belarbi [b,*], Essaid Ait Barka [a]

[a] *Université de Reims, UFR Sciences, B.P. 1039, Laboratoire de Biologie et Physiologie Végétales, 51687 Reims Cedex 2, France*
[b] *Université de Reims, UFR Sciences, B.P. 1039, Laboratoire de Microbiologie Générale et Moléculaire, 51687 Reims Cedex 2, France*

Abstract

Stresses applied to plants by pathogens such as fungi, bacteria, and viruses are well documented. However, to our knowledge, no study has focused on the effect of yeasts on plants. In this work the relationship between the growth of yeast, *Saccharomyces cerevisiae*, and its action on grapevine (*Vitis vinifera* L.) plantlets was studied. We observed that certain strains of *S. cerevisiae* could penetrate into the grapevine plants, bringing about a delay in the growth, or even causing the plantlets to die. We correlated this novel parasitic behavior of these strains of *S. cerevisiae* with their endopolygalacturonase activities and pseudohyphae formation. This study reports that the differences in behavior between the strains of *S. cerevisiae* are based on the filamentous forms, but that their pectolytic activities are required to invade grapevine tissues. The invasive process of the host plant has been confirmed histologically. Such yeast–plant interactions explain how *S. cerevisiae* may survive on grapevine throughout the years. The details of the parasitic relationship between *S. cerevisiae* and *V. vinifera* antlets together with these parameters are discussed. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Endopolygalacturonase; Plant–microorganism interaction; Phytopathogenicity; *Vitis vinifera*; *Saccharomyces cerevisiae*

1. Introduction

Yeasts constitute a highly versatile group of eukaryotic carbon-heterotrophic organisms that have successfully colonized natural habitats. The yeasts are taxonomically diverse and include ascomycetes and basidiomycetes. A third group, the imperfect yeasts, have both ascomycetous and basidiomycetous affinities [1]. Most of these are saprophytes and some are well known as human pathogens. The best-known yeast is *Saccharomyces cerevisiae*, strains of which are widely used in the fermentation of wine, beer, and other alcoholic beverages and in baking. It is also found in nature on ripe fruits. However, no study has been devoted to determine their origin(s) and the mechanism for their survival on plants.

With few exceptions, plant cells are enclosed by multilayered cell walls with specialized structures that confer protection against invaders. One of the barriers against phytopathogenic fungi is the plant polysaccharide-rich cell wall. Microorganisms in general recognize pectins, under a variety of physiological circumstances, as a potential, but complex bonded carbon source. The vast majority of fungi need to breach these barriers to gain access to the plant tissues, and for this purpose they secrete a number of enzymes capable of degrading the cell wall polymers. The action of pectolytic enzymes, and in particular of endopolygalacturonase, on cell walls is the prerequisite for cell wall degradation by other enzymes such as hemicellulase and cellulase [2,3]. Thus, most phytopathogenic microorganisms are able to degrade polysaccharides found in higher plant cell walls, and consequently establish themselves in plant tissues. Endopolygalacturonases are important in pathogenicity not only because they are involved in cell wall degradation, but also because they act as an indirect elicitor of plant defense reactions through the oligosaccharides that they release [3].

Usually *S. cerevisiae* is cellular having spherical to ovoid cells, but under specific conditions, the diploid yeast can undergo a dimorphic switch and differentiate to form pseudohyphae, growing as filaments of extended and connected cells to form rough-edged colonies that invade solid

* Corresponding author. Tel.: +33 3 26 91 32 30;
Fax: +33 3 80 39 63 26.
  E-mail address: abdel.belarbi@univ-reims.fr (A. Belarbi).

medium [4]. Recently, by combining transcriptional profiling with genetics, Madhani et al. [5] determined that the MAPK pathway controls dimorphic development in *S. cerevisiae*. One of the MAPK-regulated genes is *PGLI*, which encodes a secreted enzyme that hydrolyzes polygalacturonic acid, a structural barrier to microbial invasion present in natural substrate of *S. cerevisiae*.

In view of these results, we oriented the present study to investigate the correlation between the ability of strains of *S. cerevisiae* to switch to mycelial form, their pectolytic activities and their phytopathogenicity. To accomplish this task, we used three strains of *S. cerevisiae* (*S.c.* sp., SCPP, and Σ1278b) possessing different levels of endopolygalacturonase activity, and an endopolygalacturonase-deficient strain (BY4742 Δ*PGLI*). Such studies will bring better insight into the mechanisms by which *S. cerevisiae* survives throughout the years on the vineyard, and its potential as a plant pathogen.

2. Materials and methods

2.1. Plant material and in vitro growth conditions

Disease-free plantlets of *Vitis vinifera* L. cultivar 'Chardonnay' were obtained by growing the nodal explants on Murashige and Skoog medium [6] in 25-mm test tubes using 15 ml of medium per tube. Plantlets were grown in a growth chamber under 200 µE m^{-2} s^{-1} white fluorescent light with a 16/8-h photoperiod and a 25°C day/night temperature.

2.2. Microorganisms, growth media, cultivation

S. cerevisiae strains SCPP and *S.c.* sp. used in this work were isolated in our laboratory from fermenting Champagne wine must [7]. Yeast identification was performed according to Barnett's methods [1], and by using molecular biology techniques [8]. *S. cerevisiae* strain Σ1278b (ATCC No. 42800) was purchased from the American Type Culture Collection (LGC France SARL, Strasbourg, France). *S. cerevisiae* strains Σ1278b, *S.c.* sp., and SCPP possessed different levels of endopolygalacturonase activity (Table 1). As a control strain, we used *S. cerevisiae* strain BY4742, *Matα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, YJR153w::kan*MX4 (EUROSCARF accession No.

Y16941), in which the *PGLI* gene encoding endopolygalacturonase was deleted. Yeasts were grown in YPD medium (1% yeast extract, 1% bacto-tryptone, 1% dextrose). Cultures were inoculated with 10^5 cells ml^{-1} and then incubated at 30°C for 3 days.

2.3. Plant inoculation

The different strains of yeast were collected by centrifugation (3000 × g for 15 min) and washed twice with phosphate buffered saline (PBS) (10 mM, pH 7.4). The pellets were resuspended in PBS, the yeast concentration was adjusted to 10^6 cells ml^{-1}, and the cell suspensions were used as inocula. About 1-cm long nodal explants, taken from 6-week-old plantlets, were immersed in the inocula for 1 min, blotted with sterile filter paper, and transplanted into culture tubes. Non-inoculated controls were dipped in PBS only. The plants were grown in the growth chamber as reported above.

The phytopathogenic effect of the different strains of *S. cerevisiae* was also determined by inoculating 6-week-old plantlets. Plantlets were divided into several groups to which 10^6 cells from the different strains were deposited on the surface of the second leaf from the top.

2.4. Plant viability evaluation

The viability of plantlets was determined visually and electrolyte leakage was determined 2 weeks after inoculation of the 6-week-old plantlets. The percentage of plantlet viability was calculated based on their survival after the treatment. Viability of plantlets was estimated visually by comparing healthy plantlets to plantlets challenged with different strains of *S. cerevisiae*. Electrolyte leakage tests were performed by removing leaves from the stem, rinsing several times with distilled water, and drying on filter paper. Leaves were then transferred to 50 ml tubes with 30 ml 0.4 M mannitol for 24 h at 20°C on a rotary shaker (80 rpm). The conductivity of the solutions was measured using a conductivity meter (Orion, Model 150). Aliquots of plant tissues were autoclaved at 120°C for 3 min, cooled to room temperature and the volumes were adjusted to the initial volume. The results were expressed as percentage of total electrolytes according to Ait Barka and Audran [9]. For each experiment, 24 plantlets were used for each treatment.

Table 1
Characteristics of yeast strains used in this study

Strains	Relevant characteristics	Ref.
SCPP	Wild-type strain isolated in our laboratory. It expresses all three pectinolytic activities: polygalacturonases, pectin methylesterases, and pectate lyases	[7]
'278b	Basic endopolygalacturonase activity	ATCC No. 42800
∴ BY4742 Matα Δ*PGLI*	Used as control because the endopolygalacturonase activity was suppressed by deleting the *PGLI* gene	EUROSCARF accession No. Y16941
S.c. sp.	Wild-type strain isolated in our laboratory. It expresses a high endopolygalacturonase activity	Present study

2.5. Plate method for polygalacturonase activity

Cultures from the different strains of S. cerevisiae, adjusted to cell concentrations of 10^5 cells ml^{-1}, were dropped onto pectin-solid medium (1% pectin from apples; degree esterification <5% (Sigma, France), 0.67% Yeast Nitrogen Base, 1% dextrose, 0.6% agarose and 50 mM phosphate buffer (pH 5.5)). After 3 days of incubation at 30°C, the polygalacturonase activity was monitored on agarose plates using ruthenium red solution (0.1%) [7,10]. Plates were immersed with NaOH solution (0.2%) for 2 min to increase the staining contrast.

2.6. Microscopic preparation

Microscopic preparations were performed as described by Pottu-Boumendil [11]. From the different treatments (control and yeast-inoculated), internodal sections were cut to 1-mm pieces from six plantlets. The specimens were then immersed in cold fixative solution composed of 8% glutaraldehyde, 2% paraformaldehyde in 0.2 M potassium buffer (pH 7.24), then vacuum-filtered for 20 min and immersed in fresh fixative solution for 20 h. Samples were subsequently washed with 0.2 M potassium buffer (pH 7.24), post-fixed for 4 h in 2% osmium tetroxide prepared in the same buffer, again washed with buffer, then dehydrated in a graded ethanol series. The specimens were then washed with an acetone series and embedded in araldite (Fluka, France). Sections were stained with bromophenol blue, and examined under an optical microscope (Olympus model BH-2, Japan).
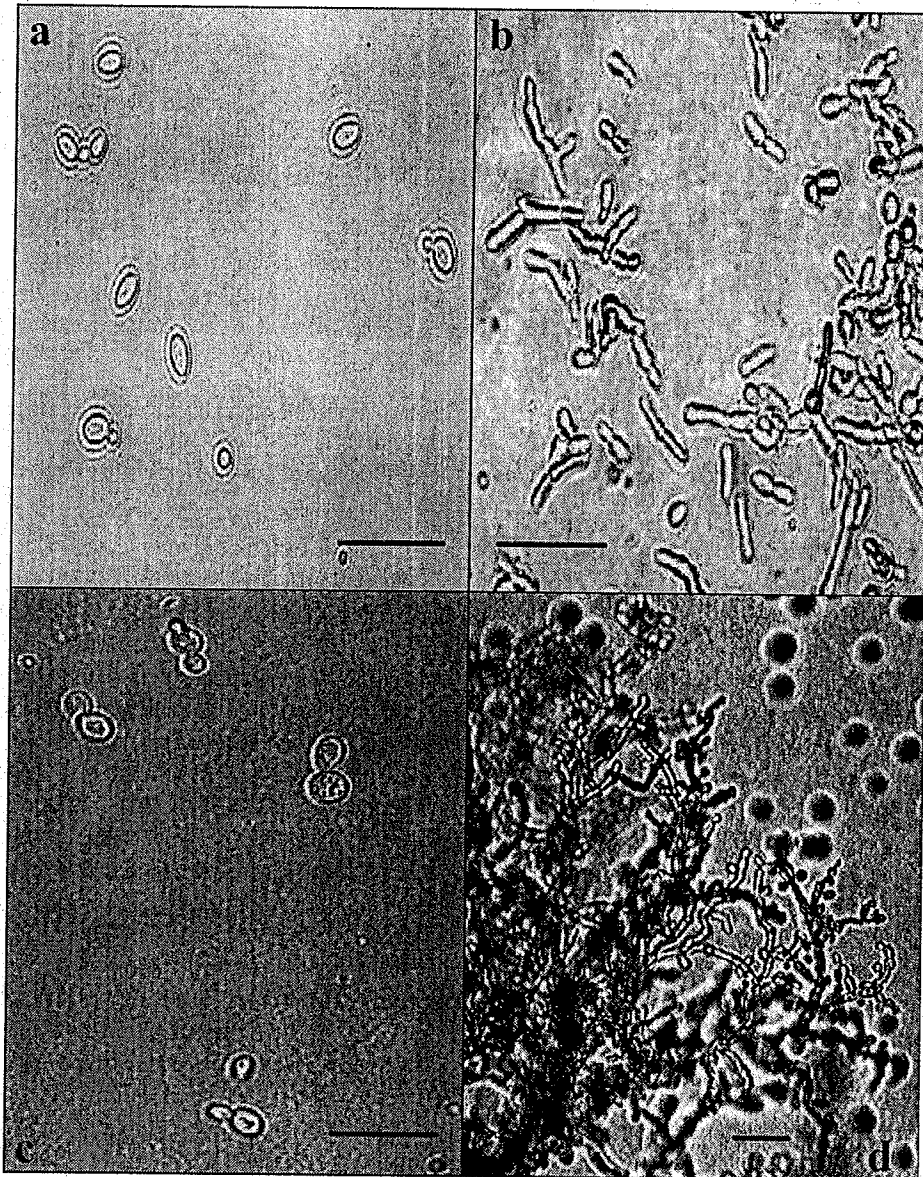

Fig. 1. Morphology of colonies produced by S. cerevisiae. a,b: Strain S.c. sp.; c,d: strain SCPP. Both strains were able to undergo a dimorphic switch and differentiate from a unicellular form (a–c) to a filamentous form (b–d). Scale bar = 10 µm (a–c), 20 µm (d).
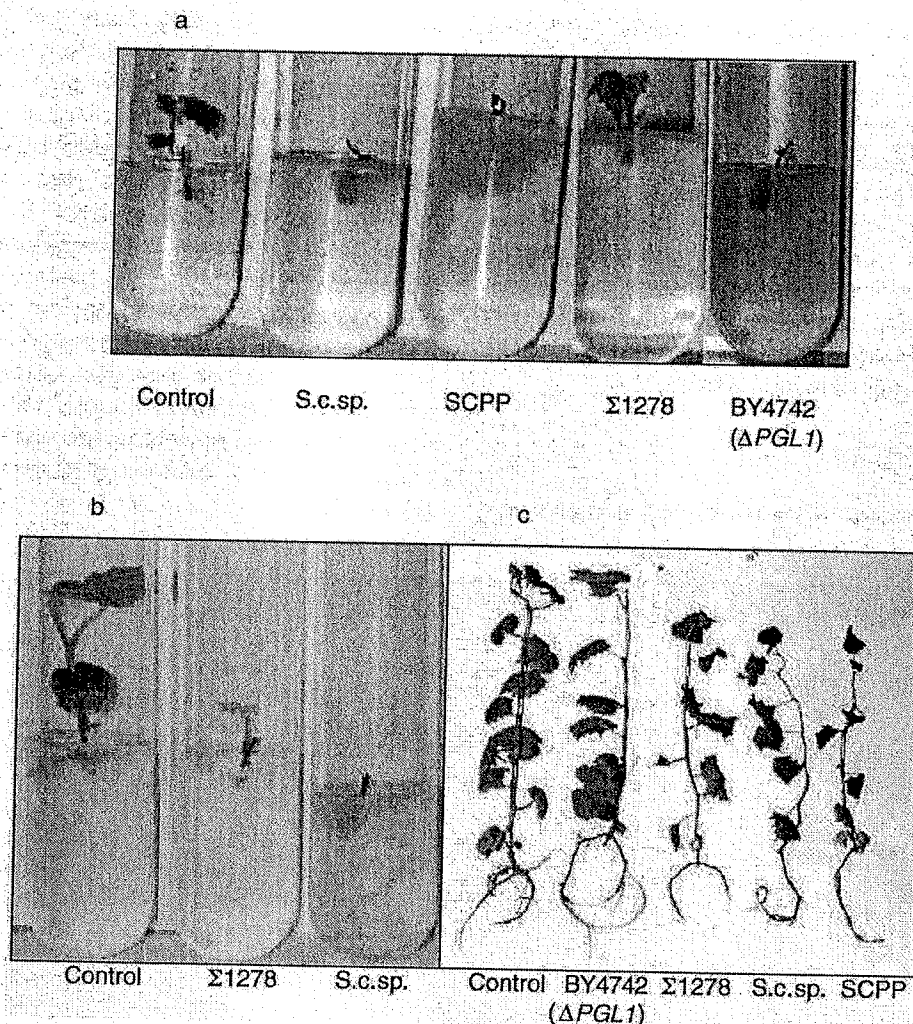
Fig. 2. Growth of internodal portion of grapevine (*V. vinifera*) plantlets inoculated with different yeast strains 10 days after inoculation (a) and 3 weeks after inoculation (b). c: 6-week-old plantlets inoculated with different yeast strains.

3. Results and discussion

Under conditions of nitrogen starvation on solid medium, some strains of *S. cerevisiae* switch their growth from spherical cells to pseudohyphae that constitute elongated cells remaining attached to each other [5]. The *S.c.* sp. and SCPP strains were able to switch to the filamentous form (Fig. 1). However, this phenomenon was not exhibited by Σ1278b and BY4742 strains (data not shown). The ability to switch between a cellular and a filamentous form has been postulated to contribute to the virulence of several fungi [5]. This switch to pseudohyphal growth results not only in the production of multicellular filaments but also in a change in the plant-host interactions [5].

The presence of strains of yeasts in the vicinity of the plantlets induced either a delay in the growth or the death of plantlets depending upon the yeast strain used (Fig. 2). Thus, strains *S.c.* sp. and SCPP caused death of the plantlets, whereas strain Σ1278b triggered a delay in their growth. The difference between the control plants and the plants inoculated with Σ1278b became very obvious

3 weeks after inoculation (Fig. 2b). Surprisingly, no growth was observed in the plantlet inoculated with strain BY4742 ΔPGLI (Fig. 2a). However, a close observation revealed the existence of a thick film around the stem in the zone of contact between plant and the cells. This film prevented the uptake of nutrients by the stem from the medium thus causing the death of the plant (Fig. 2a). Therefore, strain BY4742 ΔPGLI may induce a suffocation of the plant by the thick film formed around the plant stem. The film observed with strain *S.c.* sp. appeared 1 week after inoculation, and only 2 days around plants inoculated with strain BY4742 ΔPGLI (data not shown). The delay noted in the appearance of the thick film around the stem when they were challenged with *S.c.* sp. may be due to the fact that the majority of yeast cells invade the plant tissues, while only a small portion of yeasts is present in the medium. Thus, the growth of yeasts on medium is retarded because of the low yeast concentration at this level.

When leaves of 6-week-old plantlets were inoculated with strain BY4742 ΔPGLI, they exhibited normal growth,

whereas inoculation with the other strains delayed the growth (Σ1278b) or triggered necrosis of the plantlets (SCPP and S.c. sp.) (Fig. 2c). This observation supports the nutrient uptake hypothesis proposed for growth inhibition as an explanation for the effect of BY4742 ΔPGLI on plantlets (Fig. 2a).

No clear distinction could be made between electrolyte leakage in control plantlets and plantlets inoculated with BY4742 ΔPGLI (Table 2). The low conductivity of plants inoculated by the endopolygalacturonase-deficient strain (BY4742 ΔPGLI) confirms the non-pathogenic effect of this strain. However, the specific conductivity increased dramatically for plants inoculated with strains SCPP or S.c. sp., indicating that these strains induce the plantlets' necrosis. Results from the visual viability test support this observation (Fig. 2a). Ion leakage gave an indication that the plasma membrane integrity of plant cells was affected by the treatment. It is not certain, however, whether the cell membrane is the initial target of pathogen toxins and enzymes and that the accompanying loss of electrolytes is due to the initial effect of changes in cell membrane permeability, or whether the pathogen products actually affect other organelles or reactions in the cell, in which case cell permeability changes and loss of electrolytes are secondary effects of the initial events. If pathogens do, indeed, affect cell membrane permeability directly, it is likely that they bring this about by stimulating certain membrane-bound enzymes, such as ATPases, which are involved in the pumping of H$^+$ and K$^+$ through the cell membrane, by interfering with processes required for maintenance and repair of the fluid film comprising the membrane, or by degrading the lipid or protein component of the membrane by pathogen-produced enzymes [12,13].

In this study we found that some of the strains of S. cerevisiae affect the viability of grapevine plantlets. In order to explain the mechanism of this attack, one of the suggested hypotheses was that yeast aggressiveness is related to its ability to develop mycelial forms. However, this hypothesis is not the only one responsible for the pathogenicity since both strain SCPP and strain S.c. sp. also exhibit pseudohyphal growth, despite having different levels of aggressiveness on stem growth. When cultured on polygalacturonic acid containing agarose, the different strains exhibited different levels of endopolygalacturonase activity (Fig. 3). Thus the differences between the phyto-pathogenicity of the different strains of S. cerevisiae are based on both their filamentous state and the level of their
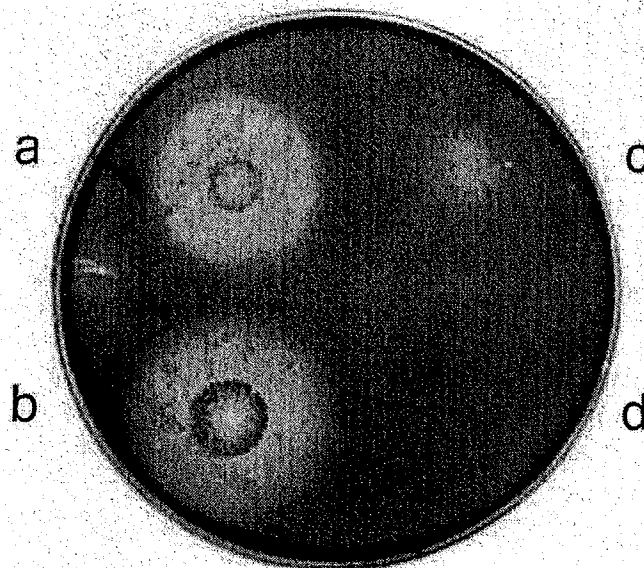

Fig. 3. Detection of polygalacturonase activity from different S. cerevisiae strains. The dark halo surrounding the colony indicates polygalacturonase activity. a: SCPP; b: S.c. sp.; c: Σ1278b; d: BY4742 ΔPGLI.

endopolygalacturonase activity. In contrast with our suggestion, Madhani et al. [5] suggested that the aggressiveness of yeast was related only to their ability to switch from isolated cells to pseudohyphal forms.

Most filamentous fungi are outfitted with a specific genetic program that allows them to infect plants and to colonize specific organs, tissues or entire plants. In order to analyze whether the yeasts did penetrate the plantlets, the inoculated plants were taken from culture media and the upper part of the stem was cut, surface-sterilized, washed and blotted with sterile filter paper. Small sections of the stem were cut and placed on petri dishes with yeast growth medium. After 2 days of incubation, the sections exhibited colonies of yeasts around them, proving that yeasts were inside the tissue of the stem of the plantlets. In contrast, no growth was observed around sectioned plantlets previously inoculated with strain BY4742 ΔPGLI (data not shown).

When a transverse sliced section of the upper stem part inoculated with different strains was observed under the microscope, plant cells inoculated with strain S.c. sp. or SCPP showed high local yeast concentrations in their tissue (Fig. 4b–d), whereas no invasion was observed in plants previously inoculated with strain Σ1278b or BY4742 ΔPGLI (Fig. 4a). These anatomical observations reveal that yeast attack is organized by a group of cells

Table 2

Percent of survival and electrolyte leakage of 6-week-old grapevine (V. vinifera) plantlets 2 weeks after their inoculation with different strains of S. cerevisiae

	Control	BY4742 ΔPGLI	Σ1278b	S.c. sp.	SCPP
Plantlet survival	100	98 ± 1.5	70 ± 3	21 ± 2	9 ± 2.5
Electrolyte leakage	10 ± 2	13 ± 1.5	42.5 ± 2.5	60 ± 3.5	70 ± 2

Data are means of four replicates; variance is represented by standard error of the mean (P≤0.05). Twenty-four plantlets were used per replicate.
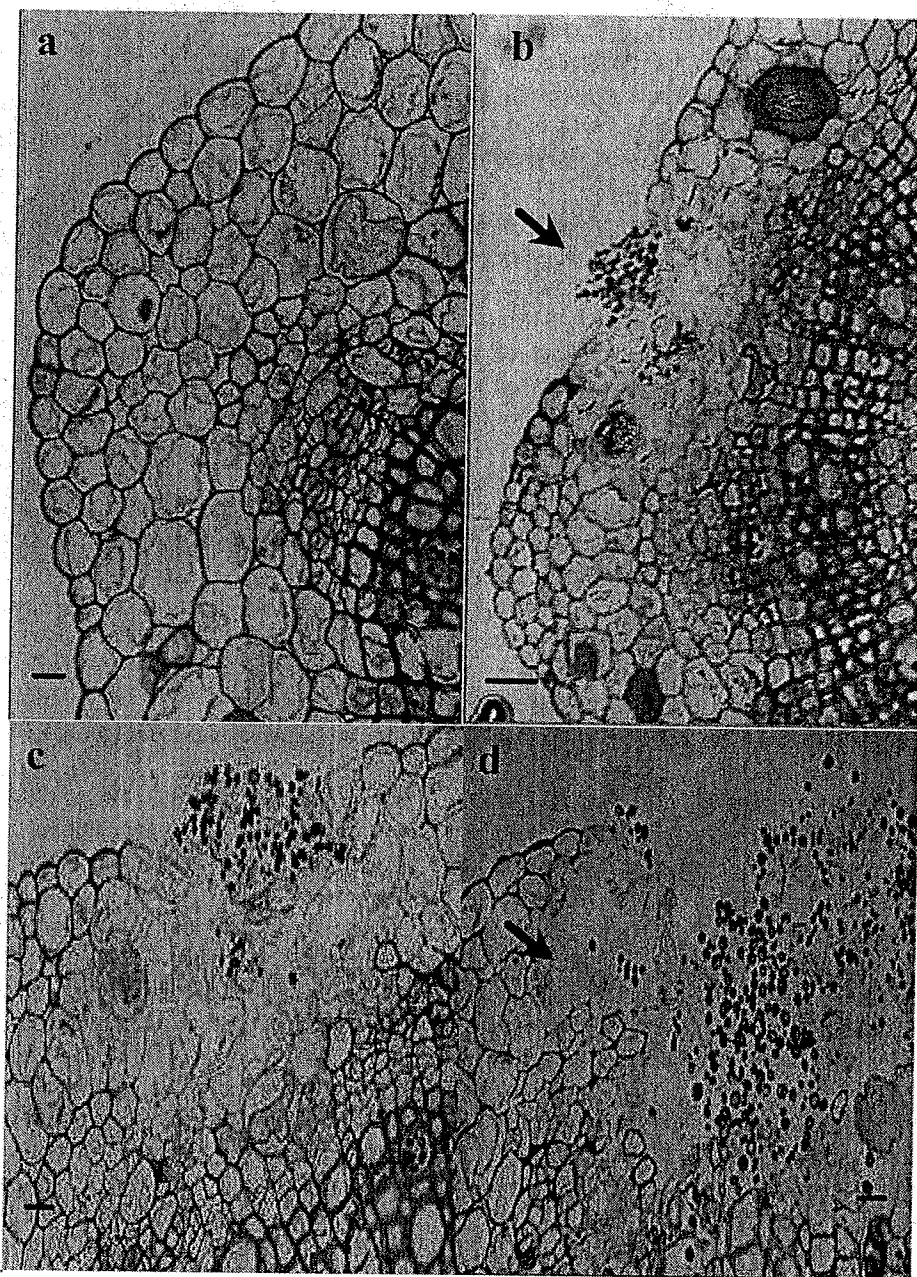
Fig. 4. Effect of inoculation with *S. cerevisiae* on stem anatomy of grapevine (*V. vinifera*) plantlets. Light micrographs of cross-sections of plantlets. a: Control; b–d: inoculated plantlets showing different levels of attack 3 weeks after inoculation. Scale bar = 20 μm.

targeting the stem first on a limited area (Fig. 4b,c). Yeast colonies penetrate then deep into plant tissues, inducing their disorganization (Fig. 4d). Studies of longitudinally sectioned stems confirmed this observation and showed that yeast colonies grew into the stem (Fig. 5a,b), and were able to establish filamentous forms (Fig. 5c,d). Due to the aggressiveness of the yeast cells, the host exhibited a necrosis around the site of penetration (Figs. 4c,d and 5b–d).

The secretion of hydrolytic enzymes by the yeast is likely an important factor responsible of their pathogenicity towards the grapevine plantlets. It is known that the ability to produce a number of polysaccharide degrading enzymes is a feature of plant pathogens and of saprophytes in the soil microflora that are responsible for the decay of plant materials [14]. Yeast strains capable of pseudohyphal growth may secrete lytic enzymes capable of hydrolyzing polysaccharides. This may be one of the reasons for the invasive habit of pseudohyphae of *S. cerevisiae* that may penetrate substrates such as grapes [5].

The microflora of grapevine varies according to the grape variety, environmental influences, soil type, fertility, irrigation and viticultural practice, physical damage caused by mould, insects and birds, and fungicide application [15]. However, there is still a lack of agreement concerning the relative contribution of wine yeast that
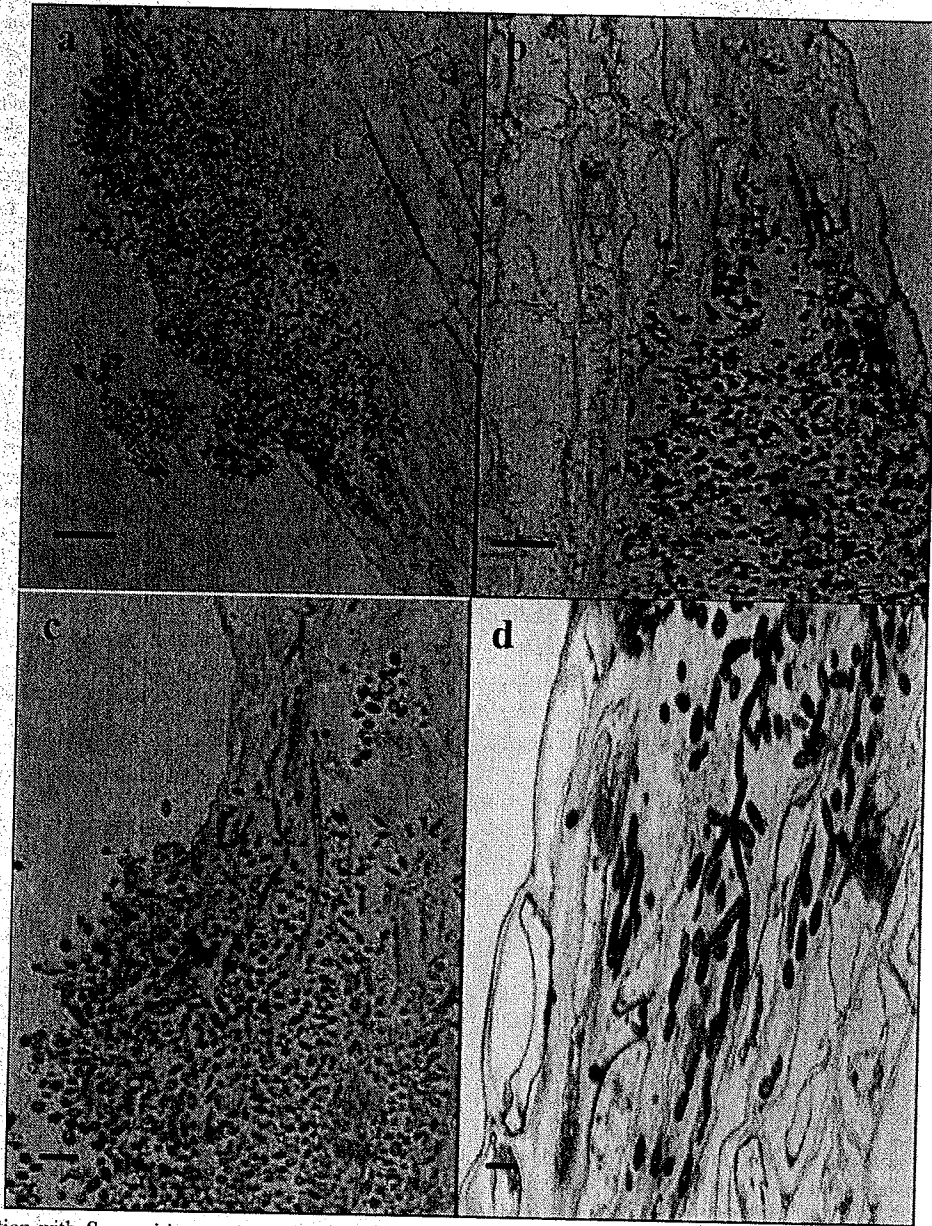
Fig. 5. Effect of inoculation with *S. cerevisiae* on stem anatomy of grapevine (*V. vinifera*) plantlets. Light micrographs of longitudinal sections of plantlets. Yeast colonies showing the disorganization of cells surrounding the zone of attack (a,b), and exhibiting a filamentous form inside the stem (c,d). Scale bar = 10 μm.

may originate in the vineyard compared to that which may originate in the cellar. It was suggested that fermentative species of *Saccharomyces* occur in extremely low populations on healthy, undamaged grapes and are rarely isolated from intact berries and vineyard soil [16,17]. Mortimer and Polsinelli [17] reported that the yeasts are brought to the berries by insects and that they multiply in the rich medium of the grape interior. Recently, Pretorious [15] reviewed the controversial origin of *S. cerevisiae* claiming that the primary source of this commercially important yeast is the vineyard, and that the presence or ʾsence of *S. cerevisiae* differs with each plant and grape cluster [18]. Others believe the evidence points to a direct association with artificial, man-made environments such as

wineries and fermentation plants, and that a natural origin for *S. cerevisiae* should be excluded [16]. Our study supports in part the first hypothesis, because *S. cerevisiae* can penetrate the plant via wounds. However, we add that strains of *S. cerevisiae* can also invade the plant tissues on their own, utilizing their arsenal of pectolytic enzymes. This explains how they survive on the plants throughout the years. In this respect, it will be interesting to demonstrate the eventual presence of yeast strains inside plants in the vineyard.

In many instances of pathogenesis by fungi or bacteria, it is an interaction between the pathogen and the carbohydrate of the host which determines the pathogen's ability to produce enzymes capable of degrading the host's cell

walls. The production of these enzymes, then, determines whether or not a successful infection will be initiated. Several studies reported that yeasts possess a complex of pectolytic enzymes [7,19]. These reports support our study suggesting the possible aggressiveness of strains of *S. cerevisiae* producing an endopolygalacturonase activity. This is not surprising since pectins are more exposed than other cell components, and consequently, pectolytic enzymes may play a major role in the penetration of plant tissues by microorganisms.

S. cerevisiae was not previously known to be a plant parasite and certainly not on *V. vinifera*. In this study, however, some strains of this yeast showed the capacity to invade the young grapevine plantlets and to either slow down their growth, or to cause necrosis and ultimately the death of the plantlets. This is the first time that strains of *S. cerevisiae* were found to exhibit aggressiveness towards *V. vinifera*. This study shows that the differences in behavior between the strains of *S. cerevisiae* are based on their ability to switch to a mycelial form, but that their pectolytic activities are required to invade grapevine tissues. Such knowledge will ultimately lead to a better understanding of the characteristics that allow *S. cerevisiae* or other pathogens to attack plants.

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